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MICROBIOLOGICAL CONTROL OF CULTURE MEDIA USED IN THE EVIDENCE OF FOOD-BORNE PATHOGENS AND THEIR PERFORMANCE PARAMETERS ON QUANTITATIVE AND QUALITATIVE METHODS

Ana-Maria TUDOSIE, Gabriel GÂJÂILĂ, Ioan Liviu MITREA, Mariana IONITA

University of Agronomic Sciences and Veterinary Medicine of Bucharest,
Faculty of Veterinary Medicine, 105 Splaiul Independenței, District 5, Bucharest, Romania

Corresponding author email: tudosieana14@yahoo.com

Abstract

A culture medium contains basic elements (water, nutrients), to which are added various supplements capable of contributing to the growth of the bacteria of interest, and at the same time, inhibiting the association flora. This study aimed for testing and optimization of special supplemented culture media used for diagnosis of microbial origin food-borne pathogens. For this, performance criteria (productivity, selectivity, and specificity) of ten different culture media used for the identification and characterization of most common bacteria (i.e., Listeria monocytogenes, Salmonella spp., Escherichia coli and Staphylococcus aureus) involved in food poisoning, were investigated. For the evaluation of the performance parameters, artificial contamination was performed with Reference Materials (MR) represented by reference strains, on two levels of contamination (low and high) and blank samples. On the qualitative methods (detection of L. monocytogenes and Salmonella spp.), selectivity, specificity, accuracy, concordance and evaluation of the detection limit at 50% (LOD50) were tested; while, on the quantitative methods (enumeration of E. coli and S. aureus), repeatability, reproducibility, critical difference and measurement uncertainty were assessed. For the qualitative methods (detection of L. monocytogenes and Salmonella spp.), the evaluated parameters showed values between 82-100% and 0.429-0.564 cfu/25 g for LOD50, respectively. For the quantitative methods (enumeration of E. coli and S. aureus), the values obtained had a measurement uncertainty between 0.24-0.26 $\log_{10}$ cfu/g). The performance criteria (productivity, selectivity and specificity) of the culture media investigated were successfully achieved. These findings on the benefits of the addition of supplements for the culture media used to diagnose food poisoning provide further evidence of the importance of additional components with the role of enrichment, stimulation, inhibition, selection and highlighting of metabolic and enzymatic equipment.

Key words*: culture media, microbiological performance, quantitative and qualitative methods, food-borne pathogens.*

INTRODUCTION

Foodborne illness (also referred to as foodborne diseases) are a problem with a strong impact on public health. They are caused by the ingestion of food contaminated with microorganisms or their toxins. Toxins or microorganisms from food cause food poisoning only if they are present in large quantities and only if they are introduced into the body orally (Barzoi et al., 1999).

There are 5 factors that cause food poisoning: bacteria, toxins, parasites and viruses. Among the bacteria involved in food poisoning are *Salmonella* spp., *L. monocytogenes*, *E. coli* and *S. aureus*, the diagnosis being based on the isolation of the pathogen causing the disease.

The culture medium is defined as a sterile nutrient medium that allows the growth and study of a microorganism. The culture media have some general characteristics: to be sterile, to have specific nutrient support for the bacteria of interest, to have a certain pH, to fulfil the physiological characteristics of the bacteria and to identify safely and specifically the metabolic and enzymatic characteristics of the bacteria subject to microbiological determination (SR EN ISO 11133:2014/A2:2020).

The evolution of bacterial cultures began with the development of the first liquid culture medium by Louis Pasteur in 1860 and the first solid medium by Koch. The advent of selective media was an important step for microbiology, making it possible to inhibit unwanted bacteria without compromising the growth of bacteria of interest (Bonnet et al., 2020).

Over the years, various studies have been done on selective enrichment broths for *Salmonella*

bacteria: Rappaport-Vassiliadis (RV) and Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn), the study confirming that RV broth is the most suitable for enrichment (Hyeon et al., 2012).
Another important step was the introduction of rabbit plasma and bovine fibrinogen into Baird-Parker (BP) agar, a medium used for the enumeration of coagulase-positive staphylococci in food (Buyser et al., 1998).
This study aimed for testing and optimization of special supplemented culture media used for diagnosis of microbial origin food-borne pathogens.

MATERIALS AND METHODS

The research was carried out in the Laboratory of Food Microbiology Department of the Sanitary-Veterinary and Food Safety Vâlcea.
As research materials, were used samples previously analyzed in the laboratory (sausages, salami, prepared meat and sheep cheese) according to the standards in force: SR EN ISO 11290-1:2017, SR EN ISO 6579-1:2017/A1:2020, SR EN ISO 16649-2/2007, SR EN ISO 6888-2/2021, SR EN ISO/IEC 17025/2018, SR EN ISO 6887-1/2017, SR EN ISO 7218:2007/A1:2014, SR EN ISO 11133:2014/A1:2018, SR EN ISO 16140-3:2021.
Other research materials were reference strains (positive, negative, partially or completely inhibited) *Listeria monocytogenes*: ATCC (American type culture collection) 35152, TCS Biosciences Ltd, Botolph Claydon, England, *Listeria innocua*: ATCC 33090, Tody Laboratories Int., Bucharest, Romania, *Escherichia coli* ATCC 25922, Tody Laboratories Int., Bucharest, Romania, *Enterococcus faecalis* ATCC 29212, TCS Biosciences Ltd, Botolph Claydon, England, *Salmonella enteritidis* ATCC 13076, TCS Biosciences Ltd, Botolph Claydon, England, *Salmonella typhimurium* ATCC 14028, Tody Laboratories Int., Bucharest, Romania, *Klebsiella pneumoniae* ATCC 13883, Tody Laboratories Int., Bucharest, Romania, *Staphylococcus aureus* subsp. *aureus* ATCC 25923, Tody Laboratories Int., Bucharest, Romania, *Staphylococcus epidermidis* ATCC 12228, TCS Biosciences Ltd, Botolph Claydon, England and culture media Half Fraser broth (DF), Oxoid Ltd, Cheshire, England, Half Fraser Selective Supplement, Oxoid Ltd, Cheshire, England, Fraser broth (F), Oxoid Ltd, Cheshire, England, Fraser Selective Supplement, Condalab, Madrid, Spain, Agar Listeria according to Ottaviani and Agosti (ALOA), Oxoid Ltd, Cheshire, England, Selective Supplement, Oxoid, Differential Supplement, Oxoid Ltd, Cheshire, England (for *L. monocytogenes*), Rappaport-Vassiliadis Soya broth (RVS), Scharlau, Barcelona, Spain, Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn), Oxoid Ltd, Cheshire, England, Novobiocin Selective Supplement, Oxoid Ltd, Cheshire, England, Iodine-iodine potassium, Scharlau, Barcelona, Spain, Xylose Lysine Deoxycholate agar (XLD), Oxoid Ltd, Cheshire, England, Brilliant Green agar (BGA), Biolife Italiana, Milan, Italy (for *Salmonella* spp.), Tryptone Bile X-Glucuronide agar (TBX), Oxoid Ltd, Cheshire, England (for *E. coli*), Giolitti-Cantoni broth (GC), Scharlau, Barcelona, Spain, Baird-Parker agar (BP), Biolife Italiana, Milan, Italy, Egg Yolk Tellurite Emulsion, Scharlau, Barcelona, Spain, Rabbit Plasma Fibrinogen (RPF) Supplement, Biolife Italiana, Milan, Italy (for coagulase-positive staphylococci).
On the qualitative methods selectivity, specificity, accuracy, concordance and evaluation of the detection limit at 50% (LOD50) were tested; while, on the quantitative methods repeatability, reproducibility, critical difference and measurement uncertainty were assessed (SR EN ISO 16140-3:2021).
Sixty samples (20 for high contamination level, 20 low level and 20 blank negative samples) were analysed by artificially contaminating a sample of sausages with *L. monocytogenes*, the reference strain ATCC 35152, TCS Biosciences Ltd, Botolph Claydon, England. The high level of contamination is represented by the 10^{-7} dilution of 1 Mc Farland ($3x10^{-8}$) and the low level by the 10^{-8} dilution of 1 Mc Farland ($3x10^{-8}$).
Sixty samples (20 for high contamination level, 20 low level and 20 blank negative samples) were analysed by artificially contaminating a sample of salami with *S. enteritidis*, the reference strain ATCC 13076, TCS Biosciences Ltd, Botolph Claydon, England.

The high level of contamination is represented by the 10^{-7} dilution of 1 Mc Farland ($3x10^{-8}$) and the low level by the 10^{-8} dilution of 1 Mc Farland ($3x10^{-8}$).

For the detection and enumeration of ß-glucuronidase-positive *E. coli* (artificially contaminating prepared meat with *E. coli* ATCC 25922, Tody Laboratories Int., Bucharest, Romania) and coagulase-positive staphylococci (artificially contaminating cheese with *S. aureus* subsp. *aureus* ATCC 25923, Tody Laboratories Int., Bucharest, Romania) 10 samples in duplicate by 2 analysts were analysed for each method.

RESULTS AND DISCUSSIONS

This study shows microbiological control of the following culture media: Demi-Fraser broth (DF), Fraser broth (F), Agar Listeria according to Ottaviani and Agosti (ALOA), Tryptone Bile X-Glucuronide agar (TBX), Rappaport-Vassiliadis Soya broth (RVS), Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn), Xylose Lysine Deoxycholate agar (XLD), Brilliant Green Agar (BGA), Giolitti-Cantoni broth (GC), Baird-Parker agar (BP).

Based on the results obtained for *L. monocytogenes*, blank negative 0/20 detected, low level of contamination 17/20 detected and high level of contamination 20/20 detected, the performance parameters (Table 1) were evaluated, all of them fulfilled the requested conditions (Gasanov et al., 2005).

Table 1. Analyzed parameters for *L. monocytogenes*

Parameters	Low level of contamination (%)	High level of contamination (%)
Selectivity	85	100
Specificity	100	100
Accuracy	93	100
Concordance	100	100

As can be seen, LOD50 represents the highest dilution or the lowest concentration of *L. monocytogenes*, at which at least 50% of the samples are positive. At the low level of contamination 7 out of 10 samples tested were positive (70%), more than 50%. The high level of contamination is represented by the 10^{-6} dilution of 0.5 Mc Farland ($1.5x10^{-6}$) and the low level by the 10^{-6} dilution of 0,5 Mc Farland diluted 1/3. For the statistical calculation of the LOD50, the literature guidelines of Wilrich (2009) were used, the result being 0.564 cfu/ 25 g (Table 2).

Table 2. LOD50 for *Lysteria monocytogenes*

High level of contamination (4 cfu/ml)		Low level of contamination (1 cfu/ml)		BLANK NEGATIVE	
Samples	cfu/ml	Samples	cfu/ml	Samples	cfu/ml
1	6	1	0	1	0
2	4	2	3	2	0
3	5	3	1	3	0
4	4	4	2	4	0
5	5	5	0	5	0
6	3	6	2	6	0
7	5	7	3	7	0
8	6	8	1	8	0
9	4	9	0	9	0
10	5	10	2	10	0
4.7 cfu=5 ufc		1.4 cfu=1 ufc			

The evaluated performance criteria (productivity, selectivity and specificity) of the culture media, used for *L. monocytogenes* detection, were successfully achieved (Table 3).

Table 3. Performance of culture media for *Lysteria monocytogenes*

Culture media	**Productivity**	**Selectivity**	**Specificity**
Half Fraser	>10 (36) specific colonies on ALOA agar	total inhibition	-
Fraser	>10 (29) specific colonies on ALOA agar	total inhibition	-
Agar Listeria according to Ottaviani and Agosti	0.73	total inhibition	blue-green colonies without opaque halo

Based on the results obtained for *Salmonella* spp., blank negative 0/20 detected, low level of contamination 18/20 detected and high level of contamination 20/20 detected, the performance parameters (Table 4) were evaluated, all of them fulfilled the requested conditions.

Table 4. Analyzed parameters for *Salmonella* spp.

Parameters	Low level of contamination (%)	High level of contamination (%)
Selectivity	82	100
Specificity	100	100
Accuracy	95	100
Concordance	100	100

As can be seen, LOD50 represents the highest dilution or the lowest concentration of *Salmonella* spp., at which at least 50% of the samples are positive. At the low level of contamination 8 out of 10 samples tested were positive (80%), more than 50%. The high level of contamination is represented by the 10^{-6} dilution of 0.5 Mc Farland ($1.5x10^{-6}$) and the low level by the 10^{-6} dilution of 0,5 Mc Farland diluted 1/3. For the statistical calculation of the LOD50, the literature guidelines of Wilrich (2009) were used, the result being 0.429 cfu/ 25 g (Table 5).

Table 5. LOD50 for *Salmonella* spp.

High level of contamination (4 cfu/ml)		Low level of contamination (1 cfu/ml)		BLANK NEGATIVE	
Samples	cfu/ml	Samples	cfu/ml	Samples	cfu/ml
1	5	1	1	1	0
2	5	2	2	2	0
3	6	3	2	3	0
4	4	4	0	4	0
5	5	5	1	5	0
6	3	6	2	6	0
7	6	7	0	7	0
8	4	8	1	8	0
9	5	9	2	9	0
10	4	10	1	10	0
4.7 cfu=5 cfu		1.2 cfu=1 cfu			

The evaluated performance criteria (productivity, selectivity and specificity) of the culture media, used for *Salmonella* spp. detection, were successfully achieved (Table 6).

Table 6. Performance of culture media for *Salmonella* spp.

Culture media	Productivity	Selectivity	Specificity
Rappaport Vassiliadis Broth	>10 (74) specific colonies on XLD agar	<10 (5) white colonies on TSA	-
Muller-Kauffmann Tetrathionate-Novobiocin Broth	>10 (93) specific colonies on XLD agar	<10 (6) white colonies on TSA	-
Xylose Lysine Deoxycholate agar	good growth of specific colonies	total inhibition	yellow colonies
Brilliant Green Agar	good growth of specific colonies	total inhibition	yellow colonies

For the evaluation of repeatability (r) the standard deviation of repeatability (STDEVr), the coefficient of variation of repeatability (VCr), the limit of repeatability (Lr) and the conditional repeatability (rC) were determined. For the evaluation of reproducibility (R) the reproducibility standard deviation (STDEVR), reproducibility coefficient of variation (VCR), reproducibility coefficient of variation limit (LVCR), reproducibility limit (LR) and conditional reproducibility (RC) were determined. In addition to these two, the critical difference (CD) and the measurement uncertainty (U) were evaluated.

The results of the quantitative method for the detection and enumeration of ß-glucuronidase-positive *E. coli* met all proposed objectives: the coefficient of variation of repeatability meets the 20% limit, the value of conditioned repeatability is less than the repeatability limit, the standard deviation of repeatability is less than the standard deviation of reproducibility multiplied by the coefficient of 0.66, the coefficient of variation of reproducibility meets the 30% limit, the limit of the coefficient of variation of reproducibility is less than the reproducibility limit, the conditional reproducibility is less than the reproducibility limit and the difference between the result obtained in the first analysis and in the second analysis is less than the critical difference (Table 7).

Table 7. Results for *Escherichia coli*

STDEVr (log10 cfu/g)	0.08
VCr (%)	18
Lr (log10 cfu/g)	0.22
rC	0.13
STDEVR (log10 cfu/g)	0.13
VCR (%)	27
LVCR (log10 cfu/g)	0.13
LR (log10 cfu/g)	0.36
RC	0.31
CD (log10 cfu/g)	0.40
Ma1-Ma2 (log10 cfu/g)	0.14
U (log10 cfu/g)	0.26

The evaluated performance criteria (productivity, selectivity and specificity) of the culture media, used for *E. coli* enumeration, were successfully achieved (Table 8).

Table 8. Performance of culture media for *Escherichia coli*

Culture media	Productivity	Selectivity	Specificity
Tryptone Bile Agar	0.87	total inhibition	white colonies

Also, the results of the quantitative method for detection and enumeration of coagulase-positive staphylococci met all the proposed objectives: the coefficient of variation of repeatability meets the 20% limit, the value of conditional repeatability is less than the repeatability limit, the standard deviation of repeatability is less than or equal to the standard deviation of reproducibility multiplied by the coefficient 0.66, the coefficient of variation of reproducibility meets the 30% limit, the limit of the coefficient of variation of reproducibility is less than the reproducibility limit, the value of the coefficient of variation of conditional reproducibility is less than the reproducibility limit and the difference between the result obtained in the first analysis and that obtained in the second analysis is less than the critical difference (Table 9).

Table 9. Results for coagulase-positive staphylococci

STDEVr (log10 cfu/g)	0.08
VCr (%)	19
Lr (log10 cfu/g)	0.22
rC	0.08
STDEVR (log10 cfu/g)	0.12
VCR (%)	29
LVCR (log10 cfu/g)	0.13
LR (log10 cfu/g	0.34
RC	0.033
CD (log10 cfu/g)	0.41
Ma1-Ma2 (log10 cfu/g)	0.01
U (log10 cfu/g)	0.24

The evaluated performance criteria (productivity, selectivity and specificity) of the culture media, used for coagulase-positive staphylococci enumeration, were successfully achieved (Table 10).

Table 10. Performance of culture media for coagulase-positive staphylococci

Culture media	Productivity	Selectivity	Specificity
Giolitti-Cantoni Broth	>10 (31) specific colonies on BP agar	total inhibition	-
Baird-Parker Agar	0.72	total inhibition	black colonies without transparent halo

CONCLUSIONS

The results of the present study provide further evidence of the importance of additional components, added to the composition of special culture media, prepared with the role of enrichment, stimulation, inhibition, selection and highlighting of the metabolic and enzymatic equipment.

In addition, these results confirm that in order to achieve an accurate identification and a complex characterization of the metabolic and enzymatic behaviour of pathogenic bacteria involved in the production of food poisoning, analytical laboratories must acquire, depending on their experience, the most efficient culture media, additional supplements for stimulation, inhibition, selection and highlighting of specific enzymatic equipment.

It should be mentioned that in case of low contamination, with a low number of microorganisms, the use of standardized methods may produce negative results in their detection. It is therefore recommended to improve the diagnostic methods currently used.

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EXPERIMENTAL MEDICINE